Determination of Pure Carotene in Plant Tissue

A Rapid Chromatographic Method

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A RESEARCH project at this laboratory involved the accurate determination of the carotene content of many samples of fresh and dehydrated vegetable products. (Carotene referred to in this paper includes both alpha- and beta-carotene, in most cases approximately 10 per cent alpha and 90 per cent beta.) The methods commonly used at present for the determination of carotene in plant materials depend upon the phasic separation of carotene from interfering pigments. The carotene is extracted from the plant material with solvents, such as alcohol or acetone, and then usually removed from the original extractant with petroleum ether. A number of workers (*1, 3, 4, 9*) have shown, however, that the petroleum ether phase obtained from dehydrated and stored plant materials or silage may still contain, after final purification, noncarotene chromogens, which are estimated as carotene.

Several methods (*1, 2, 4*) have been published in which the noncarotene chromogens are removed by contact adsorption or by passage through a Tswett column. In these procedures it is often necessary to activate the adsorbent and to carry out preliminary phasic separation with petroleum ether from the original solvent.

A procedure has been devised at this laboratory for the determination of carotene in dehydrated plant products with an adsorbent that requires no special activation and no phasic separation. The steps in brief consist of (1) extraction with a mixture of 30 per cent acetone and 70 per cent Skellysolve B, (2) removal of most of the acetone by evaporation, (3) separation of the carotene from interfering pigments by passage through an adsorption column of activated magnesium oxide, and (4) washing with 3 to 5 per cent acetone in Skellysolve B. The adsorbent is a modified mixture of Micron Brand activated magnesium oxide No. 2641 and Hyflo Super-Cel, first introduced by Strain (*8*) for the chromatographic separation of various carotenoid pigments.

Procedure

EXTRACTION OF CAROTENE. Dehydrated plant material (1.00 gram), ground to 40-mesh or smaller, with a carotene range of 50 to 500 micrograms per gram is used for the determination. One-half or 2-gram samples should be taken if the carotene content deviates from this range. The apparatus used at this laboratory is either a 300-ml. standard taper Erlenmeyer flask with a condenser and heated on a hot plate, or a Soxhlet apparatus with a 500-ml. Soxhlet flask. The material is refluxed for half an hour with 100 ml. of a mixture of 30 per cent acetone and 70 per cent Skellysolve B in the first apparatus, or for 1 hour with 200 ml. if the Soxhlet apparatus is used, operating at a rapid siphon rate. If the former apparatus is used, the sample is filtered on a sintered-glass funnel (coarse porosity) into a 500-ml. filter flask and washed with approximately 25 ml. of the Skellysolve three or four times. The filter flask, or the Soxhlet flask, containing the extract is placed directly in a steam bath and evaporated to 25 to 50 ml. Under no circumstance should all the solvent be evaporated, since this would destroy some of the carotene. This extraction procedure removes all the carotene but not all the chlorophyll.

SEPARATION OF CAROTENE FROM OTHER PIGMENTS BY CHROMATOGRAPHIC ADSORPTION. (This laboratory is at present using similar principles in large-scale preparation of carotene.) The adsorbent used is composed of three parts of Hyflo Super-Cel and one part of Micron Brand magnesium oxide, a specially activated product readily obtained on the commercial market at low cost. A 453-gram (1-pound) batch of adsorbent is conveniently prepared by spreading the Super-Cel and magnesium oxide on a large sheet of paper and mixing by turning over the heap approximately ten times, or rotating in a ball mill with the balls omitted.

The adsorption tube is made from a test tube approximately 23 × 200 mm. with a piece of glass tubing attached to the bottom. A plug of cotton or glass wool is put in the bottom, and the adsorption tube is connected to a suction flask or a large vacuum desiccator or suction bell jar. If the two latter devices are used, the receiver may be a 100-ml. or 250-ml. volumetric flask, thus avoiding later transference of the solution to the volumetric flask. With the full vacuum of a water pump, the adsorbent is added until the height of the column is about two-thirds to three-fourths the height of the adsorption tube. All the adsorbent is firmly pressed with a plunger consisting of a glass rod and properly fitting cork, and the column is washed with approximately 50-ml. of Skellysolve, which is then discarded. The evaporated extract is poured on the wet adsorbent column, which removes all the pigments. The flask and column are washed with a mixture of 3 to 5 per cent acetone in Skellysolve B until the solvent comes through colorless. The washings may be continuous or intermittent. Usually a total of 100 ml. is necessary. Full suction must be used with all the operations.

The chlorophyll and xanthophyll are held firmly at the top of the column, as are most of the other noncarotene pigments. The carotene is washed through the column by the acetone-Skellysolve mixture. The acetone is necessary because pure Skellysolve elutes the carotene from the column too slowly to be of value in routine analysis. Occasionally a small amount of noncarotene chromogen may be slowly washed down the column. The carotene is eluted much more rapidly, however, and no interference results, particularly if the final washings are pure Skellysolve B. In most cases the adsorbent may be used a number of times before it is discarded. The whole procedure of adsorption and elution, usually run in duplicate, takes from 5 to 10 minutes.

DETERMINATION OF CAROTENE. The carotene solution is made to volume and determined in any of the usual ways. In this laboratory a Lumetron photoelectric colorimeter provided with an H-4 mercury arc light and with two Corning No. 511 filters plus a neutral gray filter or two Corning No. 511 filters plus a Corning Noviol N 038 filter were used. Since the carotene solution being estimated was relatively pure, both filter combinations gave the same results, but the latter filter set, transmitting at 440 mμ, is intrinsically more accurate. A standard calibration curve was made from S. M. A. carotene that was 90 per cent beta and 10 per cent alpha, purified according to the method of Fraps and Kemmerer (*1*). All the carotene in the samples was estimated as beta-carotene, although a small but variable fraction was alpha-carotene.

Extraction of Fresh Samples

A procedure developed by Moore and Ely (*5*) in which the Waring Blendor is used to extract carotene from fresh plant samples by means of a foaming mixture of alcohol and petroleum ether was tested, and with some slight modifications proved to be excellent.

A 10-gram sample of finely minced plant material is extracted with 150 ml. of 95 per cent ethyl alcohol and 75 ml. of Skellysolve B in a Waring Blendor for 5 to 10 minutes. More alcohol is added if the mixture does not foam. The authors found it convenient to regulate the speed of the blender with a voltage controller. The extract and finely divided plant material are transferred to a fritted-glass filter by means of a piece of wide-bore glass tubing connected to the filter with a rubber stopper. The filter is attached to a suction flask. The container of the blender is alternately washed with alcohol and Skellysolve until the filtrate running into the suction flask is colorless. It was found advisable to remove the alcohol and extracted water from the Skellysolve in a separatory funnel. One hundred milliliters of water containing about 5 grams of sodium sulfate are added to the alcohol-Skellysolve mixture, and the lower aqueous alcohol solution is drawn off. The aqueous solution is extracted three times with 30 ml. of Skellysolve. The sodium sulfate aids in clearing troublesome emulsions, which may occur from time to

TABLE I. RECOVERY OF PURE CAROTENE AFTER PASSAGE THROUGH MAGNESIUM OXIDE ADSORBENT

Extinction Stock solution	Stock solution adsorbed	Recovered %
0.500	0.510[a]	102.0
0.534	0.532	99.6
0.260	0.260	100.0
0.260	0.255	98.1
		Av. 99.9

[a] Stock solution concentrated in vacuo prior to adsorption.

time. Fifty milliliters of water are poured through the combined petroleum ether fractions, the water is drawn off, and the petroleum ether is concentrated to approximately 25 ml. The magnesium oxide is stable to small amounts of alcohol, so that the prolonged extraction of the petroleum ether with water to remove alcohol, as described by Moore (*4*), is unnecessary. A few grams of anhydrous sodium sulfate are added to the Skellysolve to remove moisture, and a small layer of sulfate is placed above the adsorbent as a precautionary measure. The rest of the procedure is the same as that described for dehydrated material.

Discussion and Results

The various steps in the procedures were carefully checked.

CAROTENE RECOVERY. A purified sample of 90 per cent beta and 10 per cent alpha-carotene was used for carotene recovery tests. An aliquot of a stock solution was made to a 100-ml. volume and read in the colorimeter. A similar aliquot was washed through the adsorption column with 3 to 5 per cent acetone in Skellysolve B, made to volume, and read. In other cases the aliquot was made to 100 ml., concentrated in vacuo, and washed through the adsorbent.

The results are shown in Table I.

The recovery of carotene added to samples of dehydrated, stored pea and lima bean vines that had been shown to contain considerable noncarotene chromogens is shown in Table II. These results show that by the extraction and adsorption procedure outlined quantitative recovery of carotene may be obtained both with pure carotene (Table I) and with carotene added to plant samples (Table II).

Effect of Evaporation on Carotene. Data on the effect of vacuum versus steam concentration of the petroleum ether extracts are given in Table III. Carotene is not affected by evaporation on a steam bath under the experimental conditions.

COMPARISON OF THE ADSORPTION METHOD WITH PHASIC PROCEDURES. The determination of carotene in dehydrated and fresh or frozen plant materials by the adsorption method was compared with the widely used Peterson-Hughes-Freeman (*7*) technique as modified by Peterson (*6*). After the carotene was determined in the Skellysolve extract obtained by the Peterson-Hughes-Freeman method, an aliquot of the same solution was passed through the magnesium oxide absorbent column. Other samples were analyzed directly by the method described here. In this way a comparison of the extraction and determination of carotene by the new technique could be obtained, since the Peterson-Hughes-Freeman technique and its modifications give excellent extraction of carotene. The results shown in Table IV were obtained on samples dehydrated and stored for different periods of time, on others dehydrated and analyzed immediately, and on fresh and frozen samples.

The results in Table IV are typical of those obtained on a large number of samples. It is apparent that the phasic method records as carotene a considerable amount of noncarotene material, since on passing the extracts through the magnesium oxide column lower values are always obtained.

TABLE II. RECOVERY OF CAROTENE ADDED TO DEHYDRATED PLANT SAMPLES

Carotene in Sample	Carotene Added	Carotene Theoretical	Carotene Found	Recovered
γ	γ	γ	γ	%
72.6	114.3	186.9	190.0	101.5
8.6	114.3	122.9	122.8	100.0
37.2	114.3	151.5	152.2	100.5
62.8	114.3	177.1	171.5	96.8
77.9	114.3	192.2	190.0	98.8
				Av. 99.5

TABLE III. EFFECT OF EVAPORATION ON CAROTENE

Dehydrated Sample	Carotene Vacuum	Carotene Steam
	Micrograms per gram	
Savoy cabbage leaves	280.0	282.0
Broccoli leaves	281.0	270.0
Carrots (vacuum-dried)	486.0	486.0
Carrots (air-dried)	70.4	71.4

TABLE IV. CAROTENE CONTENT OF DEHYDRATED AND FRESH VEGETABLE PRODUCTS

(Determined by the Peterson-Hughes-Freeman technique and by the adsorption procedure)

	Carotene Found		
	PHF procedure	PHF procedure followed by adsorption	Adsorption procedure
	Micrograms per gram		
Dehydrated samples			
Pea vines	73.6	47.0	46.2
Broccoli leaves and petiole	259.0	186.0	192.0
Hubbard squash rind	68.7	39.6	40.0
Pepper	45.7	20.4	20.4
Lima bean leaves	209.0	164.0	170.0
Lima bean vines, laboratory dried	76.0	61.3	59.3
Lima bean vines, commercially dried	53.2	27.8	26.0
Asparagus tops	379.0	334.0	310.0
Spinach	878.0	828.0	810.0
Fresh or frozen samples			
Lima bean silage	36.8	21.2	21.2
Frozen savoy cabbage leaves[a]	372.0	293.0	292.0
Frozen lima bean leaves[a]	363.0	344.0	348.0
Frozen carrots and peas	94.0	...	95.5
Fresh spinach	87.8	76.5	74.3

[a] Results expressed on dry-weight basis.

These lower values agree very well with those obtained by the present method. The adsorption procedure not only removes chlorophyll and xanthophyll but also the less common pigments, such as lycopene and the noncarotene pigments of peppers, squash, and pumpkin. Samples that had been dehydrated and stored showed a considerably greater proportion of noncarotene pigments than dehydrated samples analyzed immediately. Samples stored for 3 to 6 months at room temperature lost large amounts of carotene, some undoubtedly being converted into noncarotene pigments, which are estimated as carotene by phasic procedures. Moore (*4*) and Fraps and Kemmerer (*1*) have presented similar results. The adsorption procedure also removes considerable noncarotene impurities from silage. On the whole the results of the phasic procedures show better agreement with those of the adsorption technique when fresh and frozen vegetable materials are used than on dried or ensiled samples.

A comparison of the values obtained by the magnesium oxide adsorbent with those obtained by the Baker's C. P. analyzed dicalcium phosphate adsorbent described by Moore (*4*) indicates that the adsorbents would give identical results. In many cases, however, it was necessary to readsorb the eluate that had passed through the dicalcium phosphate in order to remove small amounts of chlorophyll. In addition, the dicalcium phosphate at times may have to be specially activated in the laboratory. It is very susceptible to traces of polar solvents and much more expensive than the infusorial earth–magnesium oxide mixture, so that it is not so well adapted to routine procedures.

Duplicates run by the adsorption method agree within 3 to 5 per cent. It is difficult to estimate the accuracy of the determination, since a small and variable amount of alpha-carotene and possibly, with some materials, other carotene isomers are estimated as beta-carotene. Carotene-recovery tests and comparison with other extraction procedures indicate, however, that the adsorption procedure is considerably more accurate than phasic procedures in determining the carotene content of dehydrated and stored feeds or silage and the recovery of carotene by this method is of a high order. The authors have observed that the adsorbent will qualitatively separate alpha- and beta-carotene if the column is washed with pure Skellysolve B or Skellysolve B and 1 per cent or less acetone, and that carotene from yellow corn may easily be separated from cryptoxanthin by this procedure.

Summary

A rapid, reproducible method for the determination of carotene in plant materials, either dehydrated or fresh, has been described in detail. The method for dehydrated products consists essentially of extraction of carotene with a mixture of 30 per cent acetone and 70 per cent Skellysolve B, and subsequent separation of the carotene (alpha and beta) from interfering pigments on a column of three parts of Hyflo Super-Cel and one part of Micron Brand activated magnesium oxide. This adsorbent requires no special laboratory activation and does not destroy carotene. For dehydrated materials, phasic operations are completely eliminated, thus making the method much more rapid than any of the published phasic procedures. Fresh material is extracted by modification of the Moore and Ely method in which the Waring Blendor is used. A comparison of the results obtained by the method described with those obtained by the Peterson-Hughes-Freeman method indicates that considerable amounts of noncarotene pigments are estimated by procedures based on the phasic separation of carotene from noncarotene pigments.

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Studies in Filter Photometry

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THE single barrier layer photocell is one of the most widely used instruments of this nature, and has been subject to the most varied manner of empirical calibration. There is a definite need for improvement, particularly in respect to the exactness and fundamental nature of the measurements made and being reported with these instruments. Müller (*3*) has reviewed the literature of photoelectric photometers comprehensively; hence there is no need of considering this aspect further.

The most important use of filter photometers is in colorimetric analysis. When the solution obeys Beer's law, the relationship between log transmittance and concentration is linear. The need for instrument calibration in transmittance or its logarithm is obvious. Sheard and States (*6*) have summarized the situation existing with the use of barrier layer photocell photometers when they state that failure to obey Beer's law has been ascribed more frequently to the photocell than to the lack of monochromatic illumination. Experimental work was undertaken to determine the effect of the wave band of spectral illumination on the type of analytical calibration curve obtained. The colorimetric determination of silica by means of the silicomolybdic acid reaction was used as a test method and is particularly interesting because the absorption occurs in a spectral region of minimum sensitivity.

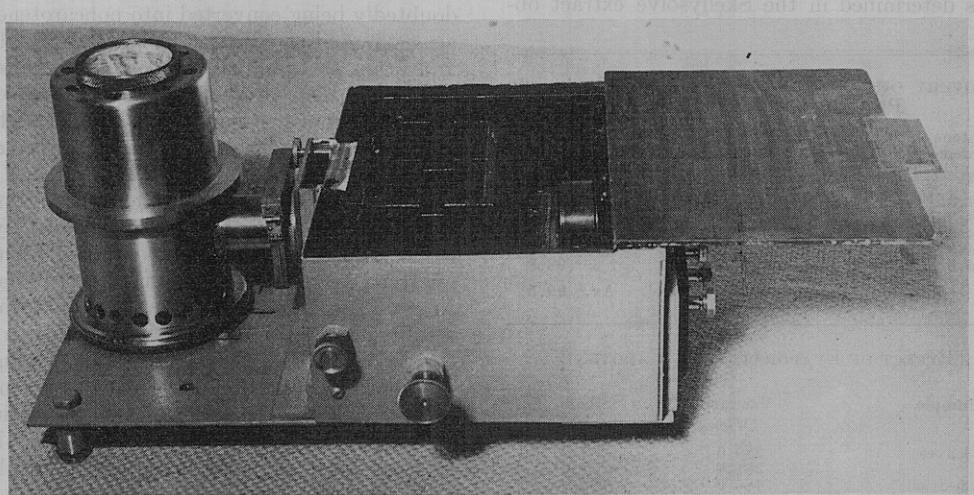

FIGURE 1. FILTER PHOTOMETER